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Flavones and Flavonols may have Clinical Potential as CK2 Inhibitors in Cancer Therapy

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ABSTRACT

The serine-threonine kinase CK2, which targets over 300 cellular proteins, is over-expressed in all cancers, presumably reflecting its ability to promote proliferation, spread, and survival through a wide range of complementary mechanisms. Via an activating phosphorylation of Cdc373, a co-chaperone which partners with Hsp90, CK2 prolongs the half-life of protein kinases that promote proliferation and survival in many cancers, including Akt, Src, EGFR, Raf-1, and several cyclin-dependent kinases. CK2 works in other ways to boost the activity of signaling pathways that promote cancer aggressiveness and chemoresistance, including those driven by Akt, NF-kappaB, hypoxia-inducible factor-1, beta-catenin, STAT3, hedgehog, Notch1, and the androgen receptor; it also promotes the epidermal-mesenchymal transition and aids efficiency of DNA repair. Several potent and relatively specific inhibitors of CK2 are now being evaluated as potential cancer drugs; CX-4945 has shown impressive activity in cell culture studies and xenograft models, and is now entering clinical trials. Moreover, it has long been recognized that the natural flavone apigenin can inhibit CK2, with a K_i near 1 micromolar; more recent work indicates that a range of flavones and flavonols, characterized by a planar structure and hydroxylations at the 7 and 4’ positions - including apigenin, luteolin, keampferol, fisetin, quercetin, and myricetin - can inhibit CK2 with K_i s in the sub-micromolar range. This finding is particularly intriguing in light of the numerous studies demonstrating that each of these agents can inhibit the growth of cancer cells lines in vitro and of human xenografts in nude mice. These studies attribute the cancer-retardant efficacy of flavones/flavonols to impacts on a bewildering array of cellular targets, including those whose activities are boosted by CK2; it is reasonable to suspect that, at least in physiologically achievable concentrations, these agents may be achieving these effects primarily via CK2 inhibition. Inefficient absorption and rapid conjugation limit the bioefficacy of orally administered flavonoids; however, the increased extracellular beta-glucuronidase of many tumors may give tumors privileged access to glucuronidated flavonoids, and nanoparticle technology can improve the bioavailability of these agents. Enzymatically modified isoquercitrin has particular promise as a delivery vehicle for quercetin. Hence, it may be worthwhile to explore the clinical potential of flavones/flavonols as CK2 inhibitors for cancer therapy.

KEYWORDS: Cancer; CK2; CX-4945; Hsp90; Flavones; Flavonols; Xenografts; Beta-glucuronidase; HDA6; Sulforaphane.

CK2 IS OVER-EXPRESSED AND UP-REGULATES PROLIFERATION, SPREAD, AND SURVIVAL IN CANCER

CK2, a serine-threonine kinase once known as casein kinase 2, is a ubiquitously expressed tetramer comprised of two catalytic subunits - α and/or α’ - and two regulatory β subunits that direct it to specific targets. CK2 is capable of phosphorylating a huge range of cellular
proteins; over 300 physiological targets have been documented to date (though ironically casein is not one of them!).\textsuperscript{1} Its level of expression and its sub-cellular localization determine its activity, as post-translational modifications or allosteric interactions are thought to have little impact in that regard; moreover, no gain-of-function mutants of this kinase are known.

Virtually all cancer cell lines studied to date overexpress CK2 protein, relative to its expression in normal tissues of origin; moreover, they tend to route a higher proportion of this protein to the cell nucleus.\textsuperscript{2} This is not likely to be accidental, as high CK2 activity works in a bewildering number of complementary ways to promote cellular proliferation and spread, while suppressing apoptosis. Hence, cancer cells which overexpress CK2 will tend to be selected for.

**CK2 MODULATES A PLETHORA OF SIGNALING PATHWAYS**

One of CK2’s most intriguing and ramified effects is to phosphorylate, and thereby activate, the co-chaperone Cdc37.\textsuperscript{3,4} Activated Cdc37 interacts with Hsp90 to provide chaperoning activity for a broad range of protein kinases, many of which play a role in promoting cell proliferation and survival. These include Akt, Src, EGFR, PDGFR, Raf-1, IKK, RIP1, Cdc2, Cdk2, Cdk4, and Cdk6. This chaperoning activity tends to slow the proteolytic degradation of these kinases, prolonging their effective half-lives; this activity is particularly crucial for the survival of certain mutant constitutively active forms of these kinases often found in cancers. To date, CK2 is the only upstream kinase known to confer activation on Cdc37 - for which reason assessment of Cdc37 phosphorylation at Ser13 has been proposed as a strategy for determining CK2 activity in vivo.\textsuperscript{5}

But CK2 works in a number of additional ways to boost the activity of signaling pathways that make cancer more aggressive and harder to kill:

**Akt**

While CK2 boosts Akt expression via Hsp90-cdc37-mediated stabilization, it can also work in various complementary ways to increase the phosphorylation and activation of this key kinase, which promotes cellular proliferation while acting in a number of ways to inhibit apoptosis. CK2 phosphorylates Akt directly at Ser129; this up-regulates the activation of Akt mediated by PDK1 and mTORC2, and facilitates its association with Hsp90.\textsuperscript{6,7} And CK2 inhibits phosphatase activities that target Akt; it phosphorylates and thereby reduces the activity of the crucial cancer suppressor PTEN, and also promotes proteasomal degradation of PML, a protein which is an obligate component of a nuclear complex that dephosphorylates Akt within the nucleus.\textsuperscript{8-11} CK2 also has the potential to work upstream from Akt, enhancing its activation by up-regulating certain tyrosine kinase signaling pathways.

**NF-kappaB**

Numerous studies show that CK2 inhibition suppresses NF-kappaB activity in cancer cell lines, whereas overexpression of this kinase boosts NF-kappaB activity.\textsuperscript{12-27} CK2 promotes degradation of I kappaB; this can reflect an activating phosphorylation of IKKbeta, as well as a direct phosphorylation of IkappaB that renders it more sensitive to proteolytic cleavage by calpain.\textsuperscript{13,14,19,24} CK2 activity also has been reported to somehow boost the expression of I KK-alpha/IKKepsilon, an alternative IkappaB kinase complex capable of promoting IkappaB degradation.\textsuperscript{17} And the transcriptional activity of p65 is enhanced by a phosphorylation of Ser529 conferred by CK2.\textsuperscript{20}

**Hypoxia-inducible factor-1 (HIF-1)**

CK2 enhances the transcriptional activity of HIF-1, even though it doesn’t increase the protein expression or nuclear binding of this factor.\textsuperscript{28,30} Some evidence suggests that this reflects a reduction of p53 levels; nuclear p53 somehow antagonizes the transcriptional activity of HIF-1.\textsuperscript{29} CK2’s impact on p53 level, in turn, may reflect phosphorylations of MDM2 that enhance its ability to promote proteasomal degradation of p53.

**Beta-Catenin**

Many studies show that CK2 inhibition decreases Wnt-beta-catenin signaling.\textsuperscript{32,33} Down-regulation of Akt, which stabilizes beta-catenin through inhibition of glycogen synthase kinase-3 and also via a direct phosphorylation on Ser552, evidently can contribute to this effect.\textsuperscript{37,38} However, CK2 also phosphorylates beta-catenin directly on Thr393, an effect which likewise prolongs the half-life and promotes the transcriptional activity of this factor.\textsuperscript{32,33}

**STAT3**

There are several reports that inhibition of CK2 suppresses STAT3 phosphorylation and activation in cancer cell lines.\textsuperscript{40,41} The basis of this effect is not yet clear. In some cell lines, suppression of IL-6 expression may contribute to this effect.

**Hedgehog**

In human lung cancer cells, CK2 activity has been shown to boost the mRNA and protein expression of Gli1, and to enhance the half-life and transcriptional activity of this key mediator of hedgehog signaling.\textsuperscript{42} CK2 can directly phosphorylate Gli1, and it has been suggested that this may be responsible for the positive impact of CK2 on hedgehog signaling.

**Notch1**

In human lung cancer cell lines expressing Notch1, in-
hinition of CK2 activity suppresses Notch1-driven transcription, whereas forced overexpression of CK2 has the opposite effect.44 This may reflect the fact that CK2 activity increases the half-life of Notch1 protein.

**Androgen Receptor**

CK2 inhibitors suppress androgen receptor-mediated transcription in prostate cancer cell lines, at least in part by blocking androgen-induced nuclear translocation of the receptor.45-47 The direct target of CK2 in this effect has not been identified.

**DNA Repair**

CK2-mediated phosphorylations of XRCC1 and MDC1, nuclear proteins which play a key role in the repair of DNA single-strand and double-strand breaks, respectively, are required for their proper activity.48-53 Hence, inhibition of CK2 can boost the killing activity of DNA-damaging cytotoxins not only by up-regulating mechanisms of apoptosis, but also by impeding the efficiency of DNA repair.

**Epidermal-Mesenchymal Transition**

Studies with CK2 inhibitors demonstrate that CK2 activity can promote the epidermal-mesenchymal transition necessary for invasive behavior by boosting expression of vimentin, snail, and smad2/3, while suppressing that of E-cadherin.54-58 Activity can promote the epidermal-mesenchymal transition necessary for invasive behavior by boosting expression of vimentin, snail, and smad2/3, while suppressing that of E-cadherin.

**NEW DRUGS FOR INHIBITION OF CK2 - CX-4945**

These considerations make it abundantly clear that well tolerated and effective pharmaceutical inhibitors of CK2 may have a bright future in oncology - both as agents for slowing cancer growth and spread, and as adjuvants to chemo- or radiotherapy. Some pharmaceutical companies are moving aggressively to evaluate the potential of this approach, and the highly potent and orally active CK2 inhibitor CX-4945 has shown impressive anti-cancer activity in mouse xenograft models, in doses which the animals appear to tolerate well.59,60 Moreover, in doses that don’t greatly retard tumor growth, CX-4945 considerably amplifies response of an ovarian cancer xenograft to gemcitabine and cisplatin - though the somewhat greater weight loss in the mice receiving combination therapy suggests that toxicity might also be increased to a degree.53 This agent is now entering clinical trials, and its progress should be followed with the greatest interest.

**FLAVONES/FLAVONOLS AS NATURAL INHIBITORS OF CK2**

However, there are other known inhibitors of CK2, one being the dietary flavone apigenin. Indeed, long before the development of the more potent pharmaceutical inhibitors of CK2, this agent was employed as a relatively specific inhibitor of CK2 in cell culture studies, with a K_i near 1 µM.64 There are indeed a number of studies, both in cancer cell culture and in mouse xenograft models, showing that apigenin can exert cancer-retardant and chemo-potentiating effects. In xenograft models, apigenin has shown activity whether administered parenterally or orally, alone or as an adjuvant to chemotherapy.65-68 Intriguingly, many of the effects of apigenin on signaling pathways reported in cell culture or xenograft studies are parallel to those of CK2 inhibition, including down-regulated activity of Akt,54-56 HIF-1,57-60 NF-kappaB,61-63 STAT3,64-66 beta-catenin,67-69 Gli1,70 AR,71-80 and Cdc37,4 and up-regulated p53.71-109 Indeed, Zhao and colleagues have recently proposed that inhibition of CK2 is a key mediator of apigenin’s anti-cancer activity in multiple myeloma cells. A survey of the burgeoning cancer research literature involving apigenin - 476 citations on Pubmed at present - reveals apigenin can influence a truly dizzying array of molecular targets in cancer cells; it is reasonable to suspect that, rather than directly inhibiting dozens of separate targets, it must be influencing one or more signaling factors that have a remarkably broad impact on the molecular biology of cancer cells. CK2 may be the crucial target in this regard. However, none of the studies in which apigenin has been administered in cancer-retardant doses to xenograft-bearing mice have assessed the impact of apigenin on tumor CK2 activity. A study assessing this - perhaps by measuring Sér13 phosphorylation of Cdc37 in tumors - would be worthwhile; and it would also be intriguing to see whether apigenin administration has any significant additional impact on cancer growth in animals that are already receiving potent doses of CX-4945; if CK2 is apigenin’s key target, little additional benefit might be seen.

Although apigenin is considered the prototype flavone inhibitor of CK2, recent studies show that other naturally-occurring flavones and flavonols have similar or slightly more potent inhibitory activity. Working in vitro with human recombinant CK2, Lolli and colleagues have recently reported that apigenin, luteolin, kaempferol, fisetin, quercetin, and myricetin can inhibit CK2 with K_i s of 0.8, 0.5, 0.4, 0.35, 0.55, and 0.92 µM, respectively.110 This inhibition is competitive with respect to the phosphodonor substrate ATP. All effective compounds are planar and are hydroxylated at the 7 and 4’ positions. Hydroxylations at 5, 3, and 3’ positions do not greatly add to or detract from activity.

These findings may help to explain the curious fact that every one of these flavones or flavonols has been reported to exert anti-cancer effects, both in cancer cell cultures, and in xenografted mice. Here are citations for the xenograft studies: apigenin,111-114 luteolin,115-122 kaempferol,123 fisetin,124-126 quercetin,127-145 myricetin.146 There are at least 53 published studies in which flavones or flavonols have decreased the growth of human xenografts in nude mice.

It seems likely that, ultimately, a drug such as CX-4945 will offer the most convenient and effective way to address the CK2 activity of clinical cancer. However, this or comparable...
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The efficacy of a given agent will presumably reflect its absorbability, the rapidity with which it is conjugated once absorbed (glucuronidation or sulfation), and its capacity to pass through cell walls. Pharmaceutical innovations which optimize absorbability might make this approach more feasible.141 With respect to quercetin, the approved food additive enzymatically-modified isoquercitrin (EMIQ), unlike quercetin, is highly soluble, but is metabolized to yield free quercetin at the intestinal brush border; a human pharmacokinetic study found that when equimolar amounts of quercetin and EMIQ were administered orally, the plasma levels of quercetin achieved were twenty-fold higher with EMIQ.147-151 This agent apparently has not yet been tested in rodent tumor models.

Rapid conjugation of absorbed flavonoids limits their capacity to exert intracellular effects.152 It is therefore fortunate that some tumors may have privileged access to flavone/flavonol glucuronide conjugates, owing to the fact that extracellular beta-glucuronidase activity tends to be elevated in tumors, particularly in their hypoxic/necrotic regions.153,154 Infiltrating immune cells may be the chief source of this activity. Moreover, the tendency of extracellular pH to be acidic in such regions can be expected to amplify their beta-glucuronidase activity.155-157 Many investigators have proposed or presented evidence that glucuronidase-masked anti-cancer agents - including flavonoids - can be selectively activated within tumors.154,157-166 Hence, the rapid glucuronidation of flavones and flavonols may not be an insuperable obstacle to the capacity of these compounds to inhibit CK2 in vivo. Perhaps this mechanism contributes to the demonstrable efficacy of flavones/flavonols in mouse xenograft studies; co-administration of a beta-glucuronidase inhibitor might clarify this. A corollary of this consideration, however, is that measurement of CK2 activity in healthy tissues following oral administration of flavones/flavonols may underestimate the capacity of these agents to inhibit CK2 within tumors.

**JOINT INHIBITION OF HDAC6 AND CK2 TO TARGET HSP90 FUNCTION**

In light of the fact that inhibition of the chaperoning function of Hsp90-Cdc37 plays a key role in the cancer-retardant efficacy of CK2 inhibitors, it is pertinent to note that acetylation of Hsp90 notably reduces its chaperoning activity.166-170 The cytosolic deacetylase HDAC6 targets these acetylations of Hsp90, restoring its activity. Hence, type II histone deacetylase inhibitors have the potential to complement the impact of CK2 inhibitors on the chaperoning of many pro-oncogenic kinases. Moreover, it has recently emerged that sulforaphane can function as an inhibitor of HDAC6 within cells;171 this phenomenon may be clinically relevant, as acute ingestion of 68 g of broccoli sprouts has been reported to suppress global histone deacetylase activity in peripheral blood mononuclear cells.169 This finding may be of particular interest, in light of the fact that HDAC6, rather like CK2, works in multifarious ways to sustain malignant cellular behavior, and is emerging as a key target for cancer therapy.172,173 It would be of interest to determine whether flavones/flavonols and sulforaphane might complement each other’s efficacy in integrative cancer therapy.

**A POTENTIAL COUNTERVAILING EFFECT - NRF2 ACTIVATION**

Although the great majority of reports examining quercetin’s impact on cancer, *in vitro* or *in vivo*, with or without concurrent chemotherapy, conclude that quercetin has cancer suppressive activity, one recent study found that, in low micromolar concentrations, quercetin protected a human ovarian cancer cell line from a range of cytotoxic drugs; concurrent quercetin administration decreased the cancer-retardant efficacy of cisplatin in a xenograft model. This effect was traced to quercetin’s ability to activate nrf2 and thereby increase the expression of antioxidant enzymes, glutathione, and glutathione-dependent detoxicant enzymes. The ability of phase 2 induction *via* nrf2 activation to promote chemoresistance in some cancers has been demonstrated. Hence, while a number of studies describe a chemosensitizing effect for quercetin in cancer models,135,136,174-176 including a report that low concentrations of quercetin sensitize some ovarian cancer cell lines to cisplatin - the possibility remains that quercetin (and presumably other phase 2-inductive flavonoids) may promote chemoresistance in some cancers. (The “flip side” of this observation is that quercetin has potential for protecting healthy tissues from chemotherapy drugs, as demonstrated in mice.177-181 These considerations, in any case, do not speak to quercetin’s potential utility as an adjuvant for slowing cancer growth.

**EVALUATING THE HYPOTHESIS**

As noted above, EMIQ may be the most appropriate agent to study in pre-clinical and clinical trials, owing to its ability to promote absorption of quercetin. In cancer xenograft models, the impact of EMIQ administration on Ser13 phosphorylation of Cdc37 in the tumor could be determined to assess this agent’s ability to suppress CK2 activity *in vivo*. Positive results in such studies could then encourage clinical cancer trials with EMIQ. Rather than expecting objective response, it would be more realistic to hope that flavonol administration will slow the growth and spread of cancer, as it does in rodent models. A placebo-controlled design might thus be required to establish clinical efficacy. The extent of Ser13 phosphorylation of Cdc37 in...
leukocytes could be measured as a surrogate for CK2 inhibition in the cancer-bearing mind, however, that quercetin metabolites might have greater activity within inflamed tumor tissue.

In regard to toxicity considerations, it should be noted that knockout of the alpha subunits of CK2 results in embryonic lethality.182,183 However, flavonols in vivo would achieve at best only partial inhibition of CK2. In rodent studies with CX-4945, cancer control is noted with doses that are not overtly toxic to the animals. Phase I clinical trials with this agent have not yet been reported, so it is not clear what the dose-limiting toxicities of CK2 inhibitors will be. Flavonols are of course prominent phytochemicals in natural diets. The toxicological evaluation of EMIQ in rodents has been described by Valentova and colleagues;147 when fed at up to 2.5% of diet to rats for 13 weeks, yellowish discoloration of bones and urine was noted, and weight gain was slightly decreased at the highest doses. At 5% of diet, isoquercetin feeding to male rats was associated with significant declines in body weight, hemoglobin, triglycerides, bilirubin, and phosphorus, with small increases in the relative weights of the lungs and testes.184 EMIQ has been accorded GRAS status for use as a food additive. These considerations suggest that it would be reasonably safe to test EMIQ in doses of several grams daily in Phase I cancer trials.

REFERENCES


39. Kim J, Hwan KS. CK2 Inhibitor CX-4945 Blocks TGF-beta1-Induced Epithelial-to-Mesenchymal Transition in A549


dietary flavonoid fisetin inhibits androgen receptor signaling and tumor growth in athymic nude mice. *Cancer Res. 2008; 68(20): 8555-8563. doi: 10.1158/0008-5472.CAN-08-0240*


145. Wong MY, Chiu GN. Liposome formulation of co-encapsulated vincristine and quercetin enhanced antitumor activity in
a trastuzumab-insensitive breast tumor xenograft model. *Nano-


147. Valentova K, Vrba J, Bancirova M, Ulrichova J, Kren V. Isoquercitrin: pharmacology, toxicology, and metabo-
fcit.2014.03.018

148. Murota K, Matsuda N, Kashino Y, et al. alpha-Oligogluc-

149. Makino T, Shimizu R, Kanemaru M, Suzuki Y, Moriwaki M, Mizukami H. Enzymatically modified isoquercitrin, alpha-
oligoglucosyl quercetin 3-O-glucoside, is absorbed more easily than other quercetin glycosides or aglycone after oral adminis-

150. Kawai M, Hirano T, Aritsui J, et al. Effect of enzymatically-


153. FISHMAN WH, ANLYAN AJ. The presence of high beta-


155. Paigen K. Mammalian beta-glucuronidase: genetics, molecu-

2796


159. Yuan L, Wagatsuma C, Yoshida M, et al. Inhibition of human breast cancer growth by GCP (genistein combined polysac-
(02)00321-4


161. Chen X, Wu B, Wang PG. Glucuronides in anti-cancer ther-


163. Murakami A, Ashida H, Terao J. Multitargeted cancer pre-

164. Legigan T, Clarhaut J, Renoux B, et al. Synthesis and antitu-
mor efficacy of a beta-glucuronidase-responsive albumin-

165. Chen KC, Schmuck K, Tietze LF, Roﬄer SR. Selective cancer therapy by extracellular activation of a highly potent gly-
cosidic duocarmycin analogue. *Mol Pharm.* 2013; 10(5): 1773-
1782. doi: 10.1021/mp300581u


ABSTRACT

Cisplatin as a highly potent cytotoxic agent was widely used in the chemotherapy of gastric cancer. It kills cancer cells by inducing apoptosis. The Extracellular signal-regulated kinase (ERK) signaling pathway plays an important role in proliferation and survival. However, its roles in apoptosis vary. This study focused on the role of ERK in cisplatin-induced apoptosis in a stomach cancer cell line, MKN-28 cells. We found that cisplatin treatment substantially activated ERK, which was prevented by MEK inhibitor U0126. Transient transfection of MKN-28 cells with Constitutively Active-MEK1 (CA-MEK1) elevated cisplatin-induced apoptosis comparing with Dominant Negative-MEK1 (DN-MEK1). Cisplatin-induced ERK activation up-regulates pro-apoptotic gene BAK, whereas down-regulates anti-apoptotic gene BCL-2. Knocking down BCL-2 with siRNA sensitizes MKN-28 cells to the toxicity of cisplatin. These results suggested that (1) ERK activation is required for the cisplatin-induced apoptosis in MKN-28 cells; and (2) ERK mediates apoptosis by BCL-2.

KEYWORDS: Cisplatin; Gastric cancer; Apoptosis; ERK; BCL-2; BAK.

INTRODUCTION

Cisplatin is a highly potent cytotoxic and genotoxic agent used in the chemotherapy for various types of cancers such as gastric cancer, which is one of the most malignant cancers in China.1-5 Apoptosis is an important pathogenic mechanism in solidary tumor,6 which is characterized by morphological changes such as cell shrinkage, chromatin condensation and fragmentation of DNA. Cisplatin treatment in vivo and in vitro results in apoptosis of cancer cells, which usually in dose- and time-dependent manner.

Several mechanisms are believed to mediate cisplatin-induced apoptosis. The primary mechanism of the cytotoxic effects is due to coordinative bonding between the atoms of platinum and DNA, leading to formation of intra-strand DNA cross-links, which induces apoptosis by activating p53 and cell cycle arrest.7 Previous study has been shown that Reactive Oxygen Species (ROS) generated by cisplatin increases lipid peroxidation, which alters enzymes and structural proteins, and direct the cell to an apoptotic pathway.7 Also, cisplatin-induced apoptosis could involve in the inflammatory pathway.9-11 Moreover, previous studies revealed that cisplatin induces apoptosis through a mechanism relaying on the Bax translocation into mitochondria and Caspase activation.12

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in the regulation of various cellular responses, such as cell proliferation, differentiation and apoptosis. Three major mammalian MAPK subfamilies have been described: the Extracellular signal-regulated kinases (ERK), the c-Jun N-terminal Kinases (JNK), and the p38 kinases. Each MAPK is activated through a specific phosphorylation cascade. The ERK pathway plays...
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Cancers in China. The functional roles of ERK in cisplatin treatment of gastric cancer remain elusive. The present study sought to delineate the role of the ERK signaling in cisplatin-induced apoptosis using a gastric cancer cell line, MKN-28.

MATERIAL AND METHODS

Cell Culture

Human gastric adenocarcinoma (MKN-28) cells were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (HyClone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 1% penicillin–streptomycin (Gibco, Grand Island, NY, USA). Cells were cultured in a 37 °C humidified incubator with a mixture of 95% air and 5% CO2.

Reagents

Constitutively Active-MEK1 and Domain Negative-MEK1 was purchased from Addgene, and then sub-cloned into pCMV vector. The MEK inhibitor U0126 and JNK inhibitor SP600125 were purchased from Tocrics (Bristol, UK). Immunoblotting experiments were performed with rabbit anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-PARP, anti-Bid, anti-BAK, anti-Bax, anti-PUMA, anti-cytochrome c and anti-BCL-2 were from Epitomics (Burlingame, CA, USA), anti-phospho-p44/42 MAPK (p-ERK1/2) (Thr202/Tyr204), and anti-ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA); The Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA).

Immunoblotting (Western Blot Assay)

Cells were lysed in PIRA buffer (Beyongtime, China). Following boiling for 5 min, samples were separated on 10% SDS-polyacrylamide gels and then transferred to an Immobilon membrane (Millipore, MA, USA). The membranes were blocked with 5% non-fat dry milk in TBS/Tween20 (0.05% v/v) for 1 h followed by incubation at 4 °C overnight with the indicated primary antibodies. The membranes were washed three times with TBST buffer and then incubated for 1 h with HRP-conjugated anti-rabbit or -mouse secondary antibodies. Visualization of protein bands was accomplished using enhanced chemiluminescence (Thermo Scientific).

Detection of Cell Apoptosis (Annexin V/PI)

Cells were seeded at a density of 1.0×105 cells per well in 24-well plates, cultured with different reagents for 48 h, and then harvested. The cells were washed with PBS and then stained with 5 μL of Propidium Iodide (PI) for 15 min followed by 5 μL of Annexin V and at room temperature in the dark according to the manufacturer’s instructions (Nanjing Key Gen Biotech, Nanjing, China). The apoptosis rate (%) of the stained cells was analyzed using FCM. The experiments were repeated three times.

Detection of Cell Death (Lactate Dehydrogenase Release Assay)

Cell death was quantitively detected based on the release of LDH by using a cytotoxicity detection kit (Roche Life Science, IN, USA) as described in manufacturer’s instruction. All the experiments were repeated three times and the average is shown in each figure.

Analysis of Cytochrome c Release

To assess mitochondrial cytochrome c release, cytosolic protein extracts were obtained according to instructions of a commercial cell fractionation kit (Enzo Life Sciences, Farmingdale, NY, USA). Briefly, 1 × 106 cells in a dish of 10 cm in diameter were washed twice with cold PBS and then centrifuged at 600g for 5 min at 4 °C. Cells were suspended in 0.1 ml of ice-cold fractionation buffer and then incubated on ice for 10 min before being homogenized with a syringe needle. The homogenates were centrifuged at 700 g for 10 min at 4 °C, after which the supernatants were collected and centrifuged at 10,000 g for 30 min at 4 °C. The supernatant was again collected as the cytosolic fraction and analyzed for cytochrome c release by western blot assay.

Silence BCL-2 with Smart Pool siRNA

SMART pool: ON-TARGET plus BCL-2 siRNA and Transfection Reagent-1 were both purchased from Damarchon (Lafayette, CO, USA), Cat# L-003307-00-0005. Transfection procedure was followed the manufacturer’s instructions.

Statistical Analysis

All results of bar graphs are expressed as the mean ± S.D. obtained from three independent experiments in duplicate. Statistical differences were evaluated using the Student’s t-test or...
ANOVA. P < 0.05 was considered statistically significant.

RESULTS

Cisplatin Induces Apoptosis in MKN-28 Cells through the Mitochondrial Pathway

Cisplatin treatment induces apoptosis in many different cell lines. To test the effect of apoptosis in MKN-28 cells, MKN-28 cells were treated with cisplatin in an increasing dose (0 μg/mL, 4 μg/mL, 8 μg/mL, 12 μg/mL, 16 μg/mL, and 20 μg/mL) for different time point (12 h, 24 h, 36 h). After the treatment, cells were stained by Annexin V-FITC and PI, and were examined by Flow cytometry (FCM). As shown in figure 1A, cisplatin treatment resulted in dose-and time-dependent manner in MKN-28 cells. Most cells were dead after 48 h treatment in dose 16 μg/mL and 20 μg/mL (Supplementary Figure 1). The apoptotic marker such as cleaved caspase-3 and cleavage of Poly (ADP-ribosyl) polymerase (PARP) can be detected by Western blot analysis (Figure 1B). The LDH releasing experiment also revealed that the LDH leaked from apoptotic cells after cisplatin treatment (Figure 1C). Meanwhile, the cell viability was opposite changed. Treatment of MKN-28 cells with cisplatin resulted in a time-dependent increase in release of cytochrome c from mitochondria (Figure 1D). These results suggested that cisplatin induced MNK-28 cell apoptosis via the mitochondrial pathway.

Cisplatin Induces Apoptosis in MKN-28 Cells with the MAPK Signaling Pathway activation

Cells were exposed in cisplatin at various time points in a dose of 20 μg/ml. The activation of MAPK pathway was then detected by Western blot, including ERK1/2 (p42/44), p38, and JNK. In the treatment, JNK was activated moderately while ERK was activated remarkably. (Figure 2) This result showed a relationship between cisplatin-mediated apoptosis and ERK signaling cascade.

ERK activation enhanced cisplatin-induced apoptosis

Pharmaceutical inhibition was applied to test if the
MAPK/ERK pathway involved in cisplatin-induced apoptosis. The effect of MAPK inhibitors was detected with Western blot (Figure 3A). Apoptosis assay analysis showed that cisplatin-induced apoptosis of MKN-28 cells was blocked by the MEK inhibitor U0126, but not c-Jun N-terminal Kinase (JNK) inhibitor SP600125 at the 24 hr and 36 hr. (Figure 3B). These results demonstrated that the cisplatin-induced apoptosis with MAPK pathway activation. It suggested that cisplatin-induced MKN-28 apoptosis was abolished by the MEK inhibitor U0126, but not by JNK inhibitor SP600125.

In addition, we test the role of ERK by ectopically expressing constitutively active and dominant-negative ERK mutant. Cells were transfected with pCMV-CA-MEK1 and pCMV-DN-MEK1 (Figure 3C). Highly activation of phospho-ERK was detected by Western blot after transiently transfected CA-MEK1, but not for the DN-MEK1 transfected cells. When these cells were treated with cisplatin, CA-ME1K1 transfected cells presented higher activation than control group while a reduced level of ERK activation appeared in dominant-negative mutant expressing cells (Figure 3D). The apoptosis assay suggested that CA-MEK1 group acquired higher ERK1 activation and apoptosis (Figure 3E and 3F). These data indicate that ERK activation induces the cisplatin-induced MKN-28 cells apoptosis.

Figure 3: Cisplatin-induced apoptosis was prohibited by MEK inhibitor, whereas enhanced by CA-MEK1 transfection. (A) Representative Immunoblot of MAPK activation within lysates generated from MKN-28 cells that were untreated or were cultured for 6 h, 12 h, 24 h, 36 h and 48 h in the presence of cisplatin (20 ug/ml).

Figure 2: Cisplatin activates the MAPK/ERK signaling pathway. Immunoblot analysis of the MAPK activation within lysates generated from MKN-28 cells that were untreated or were cultured for 6 h, 12 h, 24 h, 36 h and 48 h in the presence of cisplatin (20 ug/ml).
ERK-Mediated BCL-2 Inhibition Induces Apoptosis

To examine the relationship between ERK activation and BCL-2 family, we investigated the BCL-2 family by Western Blot. After cisplatin treatment, the BCL-2 expression level was down-regulated. Meanwhile, the BAK level was up-regulated (Figure 4A). To test if BCL-2 is the downstream transducers of the activated ERK-induced apoptosis, MKN-28 cells were transient transfected with CA-MEK. Dramatically, we found that BCL-2 was down-regulated concurrently with BAK up-regulation after CA-MEK1 transfection. The opposite result can be found after DN-MEK1 transfection (Figure 4B). This result suggested that BCL-2 and BAK are both downstream of activated ERK. Furthermore, when BCL-2 was knocked down by smart pool siRNA, cisplatin-induced apoptosis was remarkably increased (Figure 4C and 4D). Taken together, the results suggested that, with cisplatin treatment, (1) ERK activation regulate BCL-2 and BAK, (2) BCL-2 down-regulation facilitate apoptosis.

DISCUSSION

Two major distinct apoptosis pathways have been described for mammalian cells: (1) Mitochondrial independent pathway, which is recruited by the adapter molecule Fas/APO-1-associated death domain protein to death receptors upon extracellular ligand binding;23,24 (2) cytochrome c release-dependent activation of caspase-9 through Apaf-1.25-27 We did, however, observe an increase of cytochrome c in the cytoplasm of cisplatin-treated cells relative to untreated cells, suggesting that cytochrome c release is involved in cisplatin-induced apoptosis.

The MAPK pathways, including ERK, were typically studied previously. However, the roles of ERK in cancer protection are controversial.28-30 Most investigations revealed the importance of MAPK signaling pathways in regulating apoptosis during conditions of stress. But majority of the studies supported the general hypothesis that ERK activation delivers a survival signal and inhibition of ERK signaling leads to increased sensitivity of some cell lines to cisplatin.16,31,32 Although more often associated with survival, a pro-apoptotic function for the ERK pathway has also been suggested in several other model systems.22-23 In our study, however, we provided evidence that activation of ERK is important for the inducing cisplatin-induced apoptosis in MKN-28 cells. By modulating ERK activity, we found that both Domain-Negative MEK transfection and MEK specific inhibitor U0126 led to an inhibition of cisplatin-induced apoptosis. Meanwhile, enhancement of ERK activity by over-expressing Constitutively Active MEK in MKN-28 cells accentuated cell apoptosis. ERK activity-dependent cisplatin-induced apoptosis was not limited to MKN-28 cells, but also occurred in human lung cancer A549 cells and Hela cells.17,21

Although several studies have shown that JNK is activated in cisplatin treatment, their role in determining survival is unclear. A number of studies have provided evidence indicating that the JNK pathway contributes to cisplatin-induced apoptosis.15,16 However, others have suggested that JNK signal-
Concluding remarks

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We should thank all the staffs in my lab for discussing the project. This work was supported by the State Key Project on Infection Disease of China (Nos. 2012ZX10002016-004 and 2012ZX10002010-001-004). The National Natural Science Foundation of China (81202300 and 81372327).

REFERENCES


Short Communication

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Article History
Received: March 18th, 2015
Accepted: March 31st, 2015
Published: March 31st, 2015

ABSTRACT

Local recurrence in inflammatory breast cancer is common and is associated with poor prognosis. Recurrence is driven by a small population of radioresistant breast cancer cells that have stem-like properties. Previous studies have shown that high doses of the soy isoflavone genistein have a growth inhibitory effect on tumor cells, and may sensitize non-inflammatory breast cancer cells to radiation. The objective of this study was to determine the effect of genistein on the growth and radioresistance of inflammatory breast cancer cells. We show that short-term treatment of inflammatory cell lines IBC3 and SUM149 with genistein decreases cell proliferation and mammosphere forming ability only at micromolar doses, but had minimal effect on stem cell marker expression and radioresistance of these cells. However, long term treatment with a low dose (nM) of genistein, which may mimic dietary intake, decreased stem cell populations and mammosphere forming ability and increased radiation induced cell death in these lines. We propose that dietary intake of genistein may be associated with improved local response to treatment in inflammatory breast cancer by decreasing growth of breast cancer stem cells.

KEYWORDS: Inflammatory breast cancer; Genistein; Radiosensitization; Cancer stem cells.

INTRODUCTION

Inflammatory Breast Cancer (IBC) is one of the most aggressive forms of breast cancer. IBC is a rare form of breast cancer which is diagnosed according to clinical presentations, including rapid onset of erythema and edema of the skin and breast (peau d’orange). Patients with locally advanced IBC usually have a poor prognosis, with five year survival rates around 30-50%. Despite multi-modality treatments, local recurrence is common. Significantly higher incidence of loco-regional recurrences are found in IBC patients compared to those with non-inflammatory breast cancers.2

Local recurrence has been linked to the presence of radioresistant Breast Cancer Stem-like Cells (BCSC). These cells, bearing the phenotype of CD44high/CD24low-, resemble normal mammary stem cells in that they are capable of undergoing continuous self-renewal. BCSC have been shown to be resistant to radiation through several mechanisms including over expression of DNA repair enzymes, cell cycle checkpoint proteins and survival proteins. Due to their intrinsic radioresistance and self-renewal properties, BCSC are thought to promote tumor...
regrowth after radiation.

Soy isoflavones, such as genistein, have been reported to have anti-tumor properties in multiple tissue types. Treatment of non-inflammatory breast cancers with genistein has been shown to decrease proliferation and sensitize breast cancer cells to chemotherapy. Furthermore, genistein has been shown to decrease BCSC populations in non-inflammatory breast cancer. While genistein has been reported to have a radioprotective effect on normal tissues, treatment of non-inflammatory breast cancer cells with high doses (µM range) of genistein has been shown to increase radiation induced death through modulation of cell cycle and reduced expression of DNA repair proteins. Although treatment of cancer cells with high doses of genistein may have a growth inhibitory effect, dietary intake may result in long term exposure to much lower doses. The effect of chronic exposure to low doses of genistein is unclear. The aim of this study is to determine the effects of long term exposure of inflammatory breast cancer cell lines to low doses of genistein compared to short term high dose treatment.

METHODS

Cell Lines

Cell lines were purchased from Asterand Inc. (Detroit, MI, USA). SUM149 cells were cultured in Ham’s F12 medium supplemented with 5% (v/v) FBS, 1% (v/v) Antibiotic Antimycotic, 1% (v/v) Insulin-Transferrin-Selenium (ITS) and 1 µg/mL hydrocortisone (Sigma). IBC3 cells were cultured in Ham’s F12 medium supplemented with 10% (v/v) FBS, 1% (v/v) Antibiotic Antimycotic, 100 ng/mL hydrocortisone and 2 µg/mL insulin. Genistein was purchased from Sigma and was resuspended in DMSO at a stock concentration of 20 mM. All media reagents were purchased from Life Technologies unless otherwise noted.

Cell Survival Assay

Cells were plated at a density of 7000 cells per well in 96 well plates and cultured overnight. Cells were treated with genistein at the indicated concentrations and assayed for survival 72 hours later using the MTT Cell viability kit assay (Biotium Inc, Hayward, CA, USA). Data is represented as percent of untreated cells.

Mammosphere Forming Assay

Cells were removed by trypsinization and washed with culture media, followed by 3 washes with PBS. Cells were resuspended in MEM supplemented with 1X B27 (Invitrogen), 20 ng/mL Epidermal Growth Factor (EGF; Invitrogen), 20 ng/mL basic Fibroblast Growth Factor (bFGF; Invitrogen). For treatment groups, genistein was added at the indicated concentration. For long-term exposure, cells were cultured with 40 nM genistein for 2-3 weeks prior to plating for the mammosphere assay. Cells were seeded into ultra-low attachment plates (Corning Life Sciences, Salt Lake City, UT, USA) at a density of 1000-2000 cells per well. Cells were grown for 10-14 days and spheres were counted. Only spheres that were at least 50 microns or greater in size were counted. Vehicle control experiments were carried out with a dilution of DMSO only in cell culture media. Data is represented as % control spheres.

Flow Cytometry

For long-term treatments, cells were treated with 40 nM genistein in normal culture media for 3 weeks prior to the experiment. For short term treatments, cells were treated with 10 µM genistein 48 hours prior to analysis. Cells were stained with PE-conjugated CD44 and FITC-conjugated CD24 (Abcam, Cambridge, MA, USA) for 30 min at RT. Samples were centrifuged, washed, and passed through BD Falcon cell strainer cap tubes for FACS analysis. Samples were analyzed on a BD FACSAria II flow cytometer and samples were gated on an unstained control.

Clonogenic Assay

Cells were treated with 40 nM genistein for 3 weeks prior to the assay or with 10 µM genistein at the time of plating. Cells were seeded into 6 well plates at densities ranging from 50-5000 cells per well, in triplicate. Plates were irradiated at the indicated dose using an X-ray microirradiator, X-RAD225 (Precision X-ray, North Branford, CT, USA) and cultured for 10-14 days. Colonies were fixed with 10% formalin solution and stained with a 0.05% solution of crystal violet. Vehicle control experiments were carried out with a dilution of DMSO only. Individual colonies were counted and colony forming efficiency was determined as colonies counted/cells plated.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 6 software (Graph Pad, La Jolla, CA, USA). The means values of data were evaluated using ANOVA followed by an unpaired test. For all tests, P values less than 0.05 were considered to be significant.

RESULTS

Treatment of IBC Cell Lines with High Dose Genistein decreases Cell Viability

To determine the effects of high (µM) and low dose (nM) genistein on survival of inflammatory breast cancer cells we treated two cell lines, SUM149 and IBC3, with genistein and measured cell viability using an MTT-based assay 72 hours after treatment. Decreased cell survival was observed in both lines after treatment with high dose genistein (Figure 1) with a LD50 around 50 µM in the IBC3 line. SUM149 cells were slightly
more sensitive, with only 30% survival at 50 µM compared to controls. No significant effect on cell viability was observed for cells treated with doses lower than 500 nM in either short term or long term (>2 weeks). Although not significant, a slight decrease in cell doubling times was observed in cells treated with low dose genistein for greater than 2 weeks (data not shown).

**Long Term Culture of IBC Cells with Low Dose Genistein decreases Cancer Stem Cell Populations**

The ability to form mammospheres in three dimensional cultures has been associated with self-renewal and tumor initiating properties. We sought to determine the effect of high and low dose genistein on mammosphere forming ability of IBC cell lines. Cells were treated either short term or long term with genistein or vehicle carrier and plated in mammosphere forming assays. As shown in Figure 2, treatment of cells with low dose genistein at the time of plating had no effect on mammosphere formation in both lines. A significant decrease in mammosphere forming ability was observed upon treatment with high doses of genistein at the time of plating. However, the greatest decrease in mammosphere formation was observed in cells which were cultured long term with low doses of genistein, suggesting a decrease in self-renewal of these cells.

To further confirm the effects of long term exposure of IBC cells to low dose genistein on cancer stem cell populations in IBC cell lines, we examined the CD44high/CD24low/- populations in cells cultured with and without 40 nM genistein for three weeks. A significant reduction in CD44high/CD24low/- population was observed in treated cells in both lines (Figure 3). No significant changes were observed in cells treated 48-72 hours prior to analysis, even with higher doses (data not shown). This finding indicates that long term treatment with lower doses of genistein may have beneficial effects on tumor cells through a decrease in cancer stem cells.

**Figure 1:** Treatment of IBC cell lines with high dose genistein decreases cell growth and survival. MTT assay of SUM149 and IBC3 cells treated with genistein at the indicated concentrations. Cell viability was measured 72 hours after treatment. Data is reported as percent of untreated cells. Error bars represent standard deviation. Asterisk represent significance relative to untreated cells, *p <0.5, **p <.005.

**Figure 2:** Long term treatment with low dose genistein reduces mammosphere formation. SUM149 and IBC3 cells were cultured with 40 nM genistein for 3 weeks and plated in mammosphere assays. Alternatively, micromolar concentrations of genistein were added at the time of plating. Cells were grown for 10-14 days and spheres were counted. Data is expressed as % of spheres counted compared to number of spheres counted in control cells that received treatment with the vehicle control only. A significant decrease in sphere formation was observed in cells treated with long term low dose genistein. Error bars represent standard deviations. Asterisk represent significance relative to untreated cells, *p<.05, **p<.005.
Long Term Culture of IBC Cells with Low Dose Genistein increases Radiosensitivity

Previous studies have shown that breast cancer stem cell populations have increased resistance to radiation induced cell death. We therefore wanted to examine the effect of low and high dose genistein treatment of IBC cells after exposure to ionizing radiation. SUM149 cells were cultured with 40 nM genistein for 3 weeks (long term) or were treated 24 hours prior to radiation exposure with 10 µM genistein or vehicle carriers. Cells were irradiated at the indicated doses and colony formation was analyzed compared to non-irradiated controls. No differences were observed between untreated cells and cells treated with the vehicle carrier. Treatment with both 10 µM or long term treatment with 40 nM genistein decreased colony formation in non-irradiated cells (Figure 4). Combination treatment with 10 µM and ionizing radiation increased radiosensitivity at low doses of radiation, but had minimal effect on colony formation at higher doses compared to cells that were treated with radiation only. The radiosensitization effect was more significant in cells that were cultured long term with low dose genistein, even at relatively high radiation doses. This finding is consistent with the previous experiment which showed that long term culture of IBC cell lines with low dose genistein decreases stem-like populations, and it indicates that genistein may sensitize tumors to radiation through a decrease in resistant cancer stem cells.

![Figure 3: Long term treatment with low dose genistein reduces stem cell populations. IBC3 (A) and SUM149 (B) cells were cultured with and without 40 nM genistein for three weeks and analyzed for CD44 and CD24 expression by flow cytometry. A significant decrease in the stem cell population characterized by high CD44 and low CD24 expression was observed in treated cells.](image)

![Figure 4: Clonogenic assay. Genistein sensitizes IBC cell lines to radiation. SUM149 cells were cultured with and without 40 nM genistein for three weeks and exposed to ionizing radiation. Alternatively, cells were pretreated with micromolar doses of genistein or vehicle control for 24 hours prior to radiation. Cells were plated for clonogenic assay and grown for 10 days, at which time colonies were counted. Data is expressed as colony forming efficacy (log[colonies counted/cells plated]). Error bars represent standard deviations. Asterisk represents significance p < .001, relative to vehicle control cells.](image)
DISCUSSION

Local recurrence after treatment with radiation is a common event in IBC, and is associated with poor survival. Recurrence is thought to be due to the presence of radioresistant cancer stem cells. Here we show that long term treatment with low doses of genistein may help to decrease growth of cancer stem cells, and increase radiation induced cell death. Although treatment of IBC cell lines with higher doses of genistein induced cell death, minimal effect was observed on cancer stem cell populations. These findings suggest that long-term exposure may decrease proliferation of stem like populations and lead to reduced levels of resistant cells. Previous studies have shown micromolar doses of genistein increase radiation induced cell death in non-inflammatory breast cancer cell lines by induced growth arrest and accumulation of cells in the most radiosensitive phase of the cell cycle. Our data suggest that high dose genistein may act on inflammatory breast cancer cells in a similar matter, as growth arrest was detected after treatment with micromolar doses. However, this mechanism does not explain the radiosensitizing effect of long term low dose genistein as this had a minimal effect on growth of the total population of cancer cells. We propose that cancer stem cells may be more sensitive to genistein-induced growth arrest. Therefore, long-term treatment with genistein may decrease this population of radioresistant cells overtime, leading to a significant decrease in colony formation after radiation.

Genistein is a phytoestrogen that is found in soy and other legumes. Although the role of dietary soy in breast cancer has not been clear, a meta-analysis has shown that consumption of dietary soy after breast cancer diagnosis is associated with lower rates of recurrence and better survival. The components of dietary soy that are responsible for the improved prognosis have not been clearly identified. Soy food contains many components such as proteins, peptides, phenolic acids, saponins and isoflavones which may provide anti-cancer benefits. In particular, soy isoflavones such as genistein and daidzein have been directly implicated in decreases of tumor growth. While the majority of studies looking at anti-cancer properties of genistein tested concentrations at the micromolar level, our study shows that long term exposure to very low dose genistein may have an effect as well. Soy isoflavones are subjected to extensive metabolism and disposition after human digestion. Phase II metabolism by glucuronidation is a major pathway for the isoflavones, and soy isoflavones circulate in several molecular forms, including glucuronide and sulfate conjugates, freely circulating aglycons, and protein-bound aglycons. While a majority of absorbed isoflavones are excreted as conjugates into the urine, a smaller percentage undergoes enterohepatic recycling. As a result, normal physiological genistein concentration (total genistein including that from conjugated genistein after enzymatic hydrolysis) were measured as a range of 0-4192 nM with a mean of 501.9 nM from the serum of Japanese women (n=125), and ranged from 0-4092 nM with a mean of 492.7 nM in the serum of men (n=102) over forty years of age. In a recent report, the plasma concentrations of unconjugated genistein (free aglycon) in 20 healthy adults (10 women and 10 men) after consumption of soy food (soy milk, soy nuts, or Tempeh) were extremely low and no differences were observed between men and women. When all the data in that study were pooled, the mean unconjugated genistein concentration was extremely low at 0.46±0.12 ng/mL. Actual cell uptake of soy isoflavones due to dietary consumption is thus very low and might be within nanomolar level. In this study, we observed differences between long term treatments of nanomolar doses of genistein compared to acute micromolar doses of genistein. However, further study needs to be done to determine how these doses mimic long term consumption of dietary soy.

In summary, we show that treatment of inflammatory breast cancer with micromolar concentrations of genistein results in growth arrest, decreased survival, and partial radiosensitization at lower radiation doses. Long term exposure to nanomolar concentrations had minimal effect on cell growth, but it decreased stem cell populations and led to a significant decrease in colony formation following radiation treatment. Thus, our study suggests a role for long-term low dose genistein in the prevention of local recurrence, through a decrease in cancer stem cell populations. Further investigation is necessary to determine the in vivo effect of low dose genistein on stem cell populations in inflammatory breast cancer.

FUNDING ACKNOWLEDGEMENTS

This project was supported by the Delaware INBRE program, with a grant from the National Institute of General Medical Sciences - NIGMS (8 P20 GM103446-13) from the National Institutes of Health.

CONFLICTS OF INTEREST: None.

REFERENCES


Immuno-oncology: Is it a new hope for cancer patients?

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Cancer is the one of the leading causes of death, whose incidences is increasing day by day due to lack of understanding about its complete mechanism. Therefore, to understand complete mechanism of cancer, researchers started to move their focus from the cancer cell to the host and the environment in which the cancer grows, a very important component of which is the immune system. The immune system comprises of innate and adaptive system which provides protection to the body against pathogens. The immune cell receptors recognize the foreign and activate the complex immune response signaling pathways which results in the elimination of pathogens.

The first problem with cancer cells is that they arise from our own cells, so sometimes unable to recognize as non self. Further, if they recognized, alters the immune response signaling pathways at various steps which results in failure of immune response. The cancer cells can avoid recognition and elimination by altering the immune response signaling pathways at various steps such as disrupting antigen recognition and presentation mechanisms, down regulates the MHC class I molecules or inhibiting the antigen processing mechanisms. Additionally, cancer cells may disrupt the pathways which are involved in controlling T-cell inhibition and activation, or by recruiting regulatory T cells (Treg) and Myeloid-derived suppressor cells (MDSC) which are immunosuppressive. Further, cancer cells may release of some of immunosuppressive immune factors such as adenosine and prostaglandin E2, and the enzyme Indoleamine 2,3-dioxygenase (IDO) which leads to progression of cancer.

Immuno-oncology is the new and emerging field of cancer research that works to understand the interaction of the immune system with cancers cells and finds ways for harness of the patient’s immune system to treat or prevent cancer. It targets only the immune system, not the cancer cells. Researchers have been tried to understand this complex interaction for over a century, with tantalizing but unsustainable results. Recent advances in our understanding of antigen recognition, presentation and the molecules involved in T and B cell activation have provided new and excited immunotherapeutic strategies which can be used against the cancer cells. Some success in animal models has been observed and some molecules are now being under clinical trials. The interaction between our immune system and cancer is very complex process. Current strategies are based on agents that can break immune tolerance.

Presently, numbers of immunotherapy having different mechanisms for cancer patients are under clinical trials. Toll like receptors (TLRs), recognize the conserved molecular structures found in pathogens called Pathogen-associated molecular patterns (PAMPs) which leads to the activation of innate immune signaling pathways. Members of TLRs are well conserved in both human and mouse, consisting of at least 11 members. The agonists of toll-like receptors (TLRs) have been actively pursued for their anticancer potentials, either as monotherapy or as adjuvants to vaccination or other therapeutic modalities. The Bacillus calmette-guérin (BCG, an attenuated strain of Mycobacterium bovis initially developed as an anti-tuberculosis vaccine), have been shown to potently activate TLR2 and TLR4 and approved by the FDA for bladder carcinoma. Similarly, imiquimod act as TLR7 agonist and approved by the FDA for superficial basal cell carcinoma. Further, TLR9 agonists are under clinical development phase. TLR9 agonists directly induce activation and maturation of plasmacytoid dendritic
cells and enhance differentiation of B cells into antibody secreting plasma cells. The immune role of TLR9 has been studied most extensively in plasmacytoid Dendritic Cells (pDCs) and B cells, which may be the only human immune cells to constitutively express TLR9. The other most recognized class of immuno-oncology agents are checkpoint inhibitors which modulate pathways that either switch off or stimulate T cell activity, results in anticancer responses. These agents are recognized as breakthrough treatments for advanced stage cancer. The approval of sipuleucel-T (a therapeutic vaccine composed of recombinant antigen protein designed to stimulate T-cell responses) and ipilimumab, an antibody that blocks Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) were the first immunotherapies to be approved for patients with cancer. Similarly, treating patients with antibodies that block the Programmed Death-1 (PD1) receptor, or its ligand, PD-L1, has proved highly promising results in clinical trials. Cytokines have the capacity to stimulate an immune response by activating T cells development and their differentiation into the effector cells. Interleukin-2 (IL-2), a cytokine that stimulates the growth, differentiation and survival of antigen-selected cytotoxic T cells, resulted in durable anticancer responses is the first Food and Drug Administration (FDA) approval cytokine for the treatment of patients with metastatic renal cell carcinoma. IL21 and IL7 are the other cytokines which are under clinical development phase along with combination of other drugs. Another promising immunotherapy approaches is the adoptive cell transfer technology which involves the collection of T cells from patients, the in vitro expansion and activation of T cells with reactivity to cancer antigens, and the subsequent reinject back to the patient, with the expectation that the cancer-specific T cells will attack the tumor. These types of approaches are combining with other immunotherapies and are under preclinical and clinical phases which may further improve clinical efficacy.

Thus, the long term survival in cancer patients can be achieved by treating the immune system. Further, it is important now, how to use these new immunotherapies most effectively to achieve the best possible patient outcomes. Can we combine immunotherapies that target distinct immune pathways? Can we combine immunotherapeutic agent with existing treatment modalities such as radiotherapy, chemotherapy? What is the optimal dose, schedule of therapies in combination regimens? These are some important questions which have to be answered. At present, it is difficult to identify the best combination approaches, sometimes combinations leads to the unexpected toxicity (e.g. ipilimumab and vemurafenib). Thus, there is a need of more pre-clinical and clinical studies which will help to direct immunoncology research.

The development of new immunotherapies against various diseases is based upon many years of researcher’s hard work to understand the complex signaling pathways of immune systems. As that knowledge increases, researchers will hold the keys to developing new treatments that have the potential to change the ways in which we treat cancer. Although, the science of immuno-oncology is still evolving, and there are a number of important questions that remain unanswered but I hope, in future, immuno-oncology will answer most of the questions and benefit the large numbers of cancer patients with minimum side effects.

ACKNOWLEDGMENT

Author gratefully acknowledges the Department of Science and Technology (DST), New Delhi, India for providing financial assistance in the form of a DST-INSPIRE fellowship (IF 120789).

REFERENCES

10. Lu H. TLR agonists for cancer immunotherapy: tipping the balance between the immune stimulatory and inhibi-


Knowledge of the Molecular Signaling Pathways Improves the Chances of Treatment of Gastrointestinal Stromal Tumors

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INTRODUCTION

Gastrointestinal Stromal Tumors (GISTs) are the most common mesenchymal (non-epithelial) tumors of the gastrointestinal tract. A better molecular understanding of this entity, as Christopher L. Cordless in Modern Pathology in 2014 demonstrated, GISTs mainly result from two-level changes in two Oncogenes: KIT oncogene (75%) and PDGFRA oncogene (α receptor platelet-derived growth factor) which occurs in about 10% of cases; the remaining 15% are designated wild type tumors.

Having knowledge of the oncogenic pathways of this condition, allows the possibility of creating models that stratify the risk of recurrence of GIST after surgery. This risk is determined by analyzing three factors (size, mitotic index and tumor location) in very low risk patients, low risk, medium and high risk, according to the model of “NIH” (National Institutes of Health). Patients with very low risk and low-risk tumors can perform only surgery; the intermediate risk and high risk may be indicated for adjuvant treatment. Emphasis on tumors where rupture of the tumor capsule occurs, always have indication for adjuvant treatment.

Imatinib is a tyrosine inhibitor developed in the early 1990’s as a treatment chronic myelogenous leukemia due to its capacity of inhibiting the fusion oncoprotein BCR-ABL. Owing to structural similarities with KIT, several other experiments showed that imatinib can also inhibit the growth of cells that express mutant forms of KIT. This demonstrated that imatinib has a strong activity against KIT – mutant GIST cell lines.

Until then, treatment options for patients with the diagnosis of GIST was poor. However, surgery was the state of art for localized GIST. With conventional chemotherapy the response rate was less than 5% with a median survival for advanced disease approximately of 18 months. On the other hand, few data suggests that GISTS are sensitive to radiotherapy. It may have indication in a palliative situation, such as relief of symptoms, with a cumulative target dose of 30-50 Gy delivered in 2-3 Gy daily fractions.

ADJUVANT TREATMENT

In 1998, S. Hirota, et al. published an article in which the role of oncogene mutation kit and/or PDGFR in GIST was recognized. However, it was only in 2009 that imatinib was approved for the treatment of GIST expressing mutations in two oncogenes: KIT oncogene (exon 9 and exon 11) and oncogene PDGFR. This approval resulted from the American ACOSOG Z9001 trial in which 713 patients were randomized into two arms (imatinib vs. placebo); in this study there was a statistically significant impact on recurrence-free survival in the imatinib group. (Table 1)
But the European study AIO\(^6\) randomized 400 patients with operable GIST with high risk of recurrence in two groups: one received imatinib for 12 months and the other imatinib for 36 months. The evaluation at five years showed that the results for the recurrence-free survival and overall survival were more favourable in the arm of patients treated for 36 months.

**TREATMENT OF ADVANCED GIST**

In cases where a patient was treated with imatinib and developed liver metastases, one of the recommendations may be increasing the dose according to the patient’s tolerance and their comorbidities, since with this approach we may accomplish a good partial response and with a stable disease.\(^7\) (Table 2)

Another approach was analyzed in the RIGHT Study\(^8\) (Rechallenge of Imatinib in GIST having no effective Treatment – phase III study design) where new patients treated with imatinib after a first approach with imatinib or sunitinib. The results showed a significant increase in progression free survival, but no improvement in overall survival.

Sunitinib is a second-line therapy that acts in mutations in where imatinib operates (KIT oncogene mutations in exons 9 and 11), also acting in mutations of exons 13 and 14 resistant to imatinib. The work Demetri GD, et al.\(^9\) showed an improvement in progression free time of sunitinib (versus placebo).

For third-line treatments a new molecule appeared regorafenib, an oral tyrosine kinase inhibitor. Regorafenib is a multitkine inhibitor which acts against KIT, PDGFR and VEGFR. It inhibits the tumor micro-environment (PDGFR, FGRF),

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Table 2: Palliative Therapy.
proliferation of certain tumor cells (KIT, RET, RAF-1, BRAF, BRAF V600E) and also neoangiogenesis (VEGFR 1,2,3, TIE2).
Regorafenib also inhibits GIST cells with primary exon 11 mutations and secondary KIT exon 17 imatinib resistant mutations, but is less active against KIT exon 13 (V654A) mutations compared with sunitinib.

The study that led to the approval of this molecule, GRID\textsuperscript{10} (Regorafenib in Progressive Disease – phase III study design), where 199 previously treated patients with metastatic GIST unresectable were randomized into two groups: one group of patients was treated with regorafenib and best supportive care, another group was treated with placebo and best supportive care. The median progression-free survival was 4.8 months vs. 0.9 months, with clear superiority on regorafenib arm. This arm also presented a reduction of 73% in the risk of progression or death.

FUTURE

Based on pharmaco-economic studies recently published in Oncologist\textsuperscript{11,12,13}, it should be emphasized that despite the adjuvant treatment with imatinib has a significant economic impact on the national health system, its’ use allows a better approach in relation to cost-benefit level, regarding each patient. Nevertheless, the recurrence of GISTs is also associated with an economic and social cost, that are not negligible.

The optimization of targeting multiple pathways, in the treatment of GISTs will, provide a therapeutic approach aimed at the molecular tumor profile, with greater benefit for the patient and the doctor with a better global outcome.

ACKNOWLEDGEMENT

Joana Espiga de Macedo has received funds for serving as a Speaker, such as Consultant and/or an Advisory Board Member for Celgene, Merck, Pharma Mar and Roche.

REFERENCES